J. Am. Soc. Mass Spectrom. (2016) 27:669–676
DOI: 10.1007/s13361-015-1331-7

RESEARCH ARTICLE

Conformational Analysis of Proteins in Highly Concentrated Solutions by Dialysis-Coupled Hydrogen/Deuterium Exchange Mass Spectrometry

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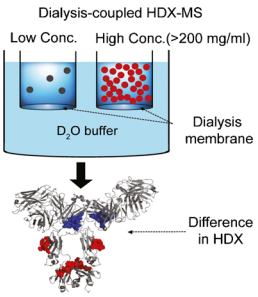

Abstract. When highly concentrated, an antibody solution can exhibit unusual behaviors, which can lead to unwanted properties, such as increased levels of protein aggregation and unusually high viscosity. Molecular modeling, along with many indirect biophysical measurements, has suggested that the cause for these phenomena can be due to short range electrostatic and/or hydrophobic protein–protein interactions. Hydrogen/deuterium exchange mass spectrometry (HDX-MS) is a useful tool for investigating protein conformation, dynamics, and interactions. However, "traditional" continuous dilution labeling HDX-MS experiments have limited utility for the direct analysis of solutions with high concentrations of protein. Here, we present a dialysis-based HDX-MS (di-HDX-MS) method as an alternative HDX-MS labeling format, which takes advantage of passive dialysis rather than the classic dilution workflow. We applied this approach to a highly concentrated antibody solution without dilution or significant sample manipulation, prior to analysis. Such a method could pave the way for a deeper understanding of the unusual behavior of proteins at high concentrations, which is highly relevant for development of biopharmaceuticals in industry.

Keywords: Hydrogen deuterium exchange, HDX, Dialysis, High concentration mAbs, Protein conformation

Received: 13 November 2015/Revised: 18 December 2015/Accepted: 24 December 2015/Published Online: 9 February 2016

Introduction

Certain clinical indications require delivery of high doses of the drug (i.e., several mg/kg) to achieve the desired bioavailability and efficacy [1, 2]. When delivered intravenously, large doses of dilute biopharmaceuticals can require very long administration times (up to several hours), and often must be administered several times per month. Such a mode of drug delivery typically necessitates multiple visits to a hospital or a doctor's office, which can be a significant burden to the patient and healthcare providers in terms of time, money, and resources. To reduce cost and enable greater patient convenience and comfort, alternative routes of administration are often preferable, such as patient self-administration via subcutaneous (SC) injection. SC injection, however, is faced with its own limitations. The maximum amount of medication that can be reasonably injected subcutaneously is approximately 1.0 to 1.5 mL. Thus, for antibody biopharmaceuticals, where doses of up to several mg/kg body weight is often desirable, highly concentrated solutions (up to several hundreds of mg/mL) may be required to deliver the necessary dose. At high concentrations, protein solutions (in the range of ≥100 mg/mL) can exhibit unwanted properties, such as increased levels of aggregation and viscosity levels that exceed the mechanical limits of syringes and autoinjector devices to deliver the dose [3–6]. As aggregation and high viscosity can affect biopharmaceutical manufacturing, efficacy, and administration route, it is critical to understand how and why these phenomena occur, in order to minimize or eliminate them [3, 7, 8]. At present, few techniques exist that can characterize biopharmaceuticals at high concentration without significant sample manipulation or dilution.

Hydrogen deuterium exchange mass spectrometry (HDX-MS) has proven to be a useful tool for investigating protein

Electronic supplementary material The online version of this article (doi:10.1007/s13361-015-1331-7) contains supplementary material, which is available to authorized users.

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conformation, dynamics, and interactions [9, 10]. However, a traditional continuous dilution labeling HDX-MS experiment has inherently limited utility for the direct analysis of proteins in high concentration solutions because it requires diluting the protein sample into the same buffer containing deuterium (i.e., D_2O instead of H_2O). HDX analysis is typically initiated with a 5- to 20-fold (or greater) dilution. Thus, if a 200 mg/mL sample is to be studied, the sample's exchange behavior at equilibrium can realistically be evaluated only at concentrations in the range between 10 to 40 mg/mL (depending on the dilution), which could negate particular high concentration effects that one wishes to measure. As a result, the use of traditional HDX labeling formats cannot be used to investigate proteins in highly concentrated solutions. Since formulating antibodies at very high concentrations is of great relevance in the biopharmaceutical industry, there is a need for novel and customized workflows for characterizing the higher order structure of proteins in order to address this gap.

In a recent study, Arora et al. used HDX-MS to study an antibody at concentrations as high as 60 mg/mL using an HDX labeling format that involved the reconstitution of a lyophilized antibody with deuterium oxide (D_2O) instead of water (H_2O) [11]. Lyophilization as a sample preparation step for HDX-MS could theoretically be performed at any protein concentration and is a dilution-free process. However, there are practical limitations that can preclude its applicability in preparing pharmaceutically relevant, highly concentrated solutions for use in HDX-MS experiments, such as the introduction of unwanted protein stresses, changes in reconstitution time, variations in viscosity, etc. [12–17]. Nevertheless, many of these challenges may be solved using alternative lyophilization strategies and protocols, but the success of this approach will depend on the protein and its specific formulation. Lyophilization is a good strategy for HDX labeling, but many biopharmaceuticals are formulated in solution and lyophilization is often not possible and/or desirable. Therefore, a dilution-free solution-to-solution based buffer exchange format (i.e., begin and end with a protein solution without altering concentration) is desirable for HDX-MS analyses of pharmaceutically relevant highly concentrated protein solutions.

Many different solution-based buffer exchange formats exist, such as ultrafiltration, size exclusion chromatography (SEC), solid phase extraction (SPE), and ion-exchange spin-filters, but these techniques change the concentration of the protein by either dilution or up-concentration. Since our goal is to perform HDX labeling on highly concentrated proteins in a simple format that does not significantly alter the concentration of the protein during labeling, these approaches were not viable options. Astorga-Wells et al. reported an alternative dilution-free HDX labeling strategy that employs a custom-designed on-line microchip setup based on an ion-selective membrane for buffer exchange and HDX labeling [18]. Although their technique is capable of avoiding sample dilution effects, the system is not commercially available and we were not certain how samples with high viscosity would behave. Hence, we pursued a simpler approach. In this work, we have used HDX-MS to investigate conformation and dynamics of proteins in an equilibrated solution at high concentrations (i.e., ≥200 mg/mL) with no dilution of the sample. We achieved this by developing an alternative HDX-MS labeling format that utilizes passive dialysis microcassettes for HDX labeling. In order to assess the feasibility of this approach, we analyzed and compared a recombinant immunoglobulin gamma 4 (IgG4) monoclonal antibody (mAb1) at high and low concentrations (3 versus 200 mg/mL). The results obtained from analysis of mAb1, along with the experimental setup and specific features of the method, are presented in this paper. This methodology can be used to provide critical insight into the unusual behavior of proteins at high concentrations.

Materials and Methods

Reagents and Antibodies

All chemicals and reagents were purchased from Sigma-Aldrich, unless otherwise noted. Deuterium oxide was purchased from Cambridge Isotope labs (Tewksbury, MA, USA) and tris (2-carboxyethyl) phosphine (TCEP) was purchased from Life Technologies (Thermo Fisher, Waltham, MA, USA). The recombinant monoclonal antibody (mAb) used in this work is an immunoglobulin gamma 4 (IgG4) and was expressed in Chinese hamster ovary (*CHO)* cells and purified at Biogen (Cambridge, MA, USA). The mAb used throughout this experiment is named mAb1 and was formulated into 50 mM sodium phosphate, 100 mM NaCl, pH 6.0 (PBS pH 6.0). Samples were concentrated to a maximum concentration of 225 mg/mL and diluted where necessary.

Continuous Dilution Labeling Hydrogen Deuterium Exchange Mass Spectrometry (HDX-MS)

Continuous labeling experiments via dilution (10 s to 14 h) were carried out as previously described [19]. Briefly, the mAb sample was incubated in deuterated buffer (pD 5.6) for various amounts of time at both 4 and 25 °C. HDX reactions were quenched by addition of 1:1 (v:v) quench buffer (7 M Guanidine HCl (GnHCl), 0.1 M citrate (pH 2.4), and 0.2 M TCEP). The samples were quickly vortexed for 30 s and immediately flash-frozen at –80 °C in liquid nitrogen until analysis. Frozen samples were rapidly thawed and diluted again 1:1 (v:v) with 0.1% formic acid prior to injection. Labeled samples were injected into a Waters nanoACQUITY with HDX Technology (Waters Corporation, Milford, MA, USA), which was held at 0.1 °C during the measurements. The protein was digested online using an immobilized pepsin cartridge (Waters Corporation) and peptides separated with a 9 min acetonitrile gradient at a flow of 40 µL/min. The separation column used was a 1.0×100.0 mm ACQUITY UPLC C18 T3 (Waters Corporation) packed with 1.7 µm particles. Deuterium levels were reported as relative deuterium incorporation [9]. All experiments were performed in

triplicate measurements. To eliminate peptide carryover, an optimized wash solution of 3 M GnHCl, 3% formic acid, and 3% acetonitrile was injected after each run [20]. Mass spectra were obtained with a Waters Synapt G2S TOF equipped with standard ESI source (Waters Corporation). The mass spectra and resulting relative deuterium levels were automatically processed and plotted with the software DynamX 3.0 (Waters Corporation). Identification of the peptic fragments was accomplished through a combination of exact mass analysis and MS^E using Identity Software (Waters Corporation) [21].

Dialysis-Based Labeling Hydrogen Deuterium Exchange Mass Spectrometry (di-HDX-MS)

Dialysis labeling was performed at 4 °C under the following conditions: 200 to 250 µL of sample was placed into a 20 kDa molecular weight cutoff (MWCO) Slide-A-Lyzer mini dialysis device with a 0.25 mL maximum volume (ThermoFischer Scientific). A small (5 × 2 mm PTFE, Teflon) stir bar (ThermoFisher Scientific) was placed inside of the microdialysis cartridge and the microdialysis cartridge was then placed into a 2–4 L beaker containing 1 L of 99.99% deuterated PBS buffer (pD 5.6), as shown in the diagram in Figure 1. Passive dialysis was performed for 3 to 24 h before the sample was removed and quenched with 7 M GnHCl, 0.1 M citrate (pH 2.4), and 0.2 M TCEP. Upon equilibrium, the dialysis conditions resulted in approximately a 4000- to 5000-fold labeling excess of deuterium. The sample was then quickly vortexed for 30 s and immediately flash-frozen at –80 °C with liquid nitrogen until analysis. Frozen samples were rapidly thawed and diluted with 0.1% formic acid prior to injection. The level of quenching and dilution was determined based on protein concentration. Protein digestion, peptide identification, separation, and mass spectrometry analysis were performed as indicated in the above section. Pymol software [22] was used to map the conformational changes to the crystal structure of an IgG1 antibody (PDB id: 1hzh).

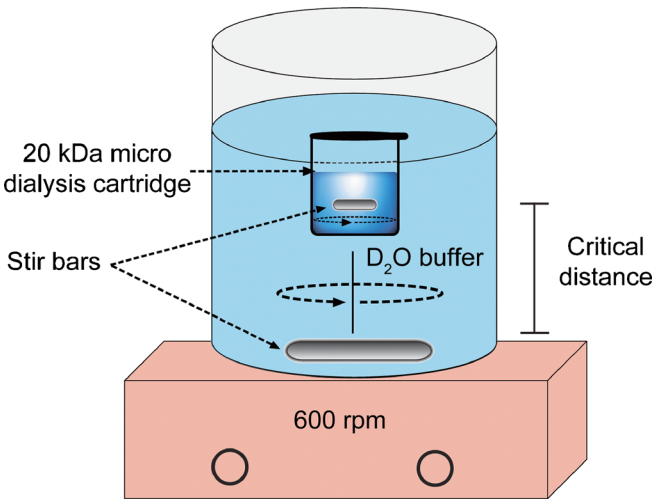

Figure 1. Schematic illustration of the dialysis-based HDX-MS experiment used for the analysis of proteins in highly concentrated solutions. Protein sample is placed inside a microdialysis cartridge, and submerged in a 2–4 L beaker containing D_2O buffer. HDX reaction is performed based on passive dialysis and after certain time points sample is quenched and injected to UPLC-MS system

Results and Discussion

Dialysis HDX Labeling Setup

The HDX labeling procedure was set up as shown in Figure 1. HDX was performed using microdialysis cartridges (250 µL maximum volume) placed into 1 L of deuterated buffer, which was continuously stirred at 600 rpm (speed will depend on stir plate and stir bar used). Dialysis was performed in a cold room at 4 °C for 3 to 24 h. For this experiment, two stir bars were used. The first was a standard 1-inch Teflon stir bar, which was placed into the 2–4 L beaker containing 1 L of deuterated PBS buffer. The second was a micro (5 × 2 mm) Teflon stir bar and was placed inside the microdialysis cartridge. Since many of the samples will be highly concentrated and may be viscous, it was necessary to place a stir bar into the microdialysis cartridge as well to ensure that the solution could properly equilibrate with the labeling buffer. The micro stir bar also helped the dialysis to reach equilibrium faster and samples could be labeled and analyzed after 1 h (these results will be published in forthcoming work); however, for the current experiments, the earliest HDX dialysis labeling time was 3 h. An important consideration for the micro stir bar is the fill height of the labeling solution from the stirring plate (shown in Figure 1). If the micro dialysis cartridge lies too high above the stirring plate, the micro stir bar will not move. Thus, it is essential to optimize the distance; the micro stir bar was placed from the stirring plate (see Video in supplemental information). The use of a 2 L beaker with 1 L of buffer appeared to provide optimal dimensions. Alternatively, smaller solution volumes can be used (e.g., 0.5 L of solution with a 1 L beaker). It should also be noted that rapid stirring of mAb solutions has been shown to induce aggregation and particle formation, particularly in highly concentrated protein solutions [23, 24]. Thus, in this study, the stirring was performed gently (see Video in Supplemental Information). In addition, the sample was analyzed after mixing with the stir bar by size-exclusion chromatography (SEC), microfluidic imaging (MFI), second order derivative UV spectroscopy, and circular dichroism (CD), and no evidence of increased aggregation, particle formation, or protein structure alteration was detected (data not shown).

Two different mini dialysis cartridges were also tested, the 20 kDa MWCO Slide-A-Lyzer from ThermoFisher Scientific and the 12 kDa MWCO Pur-A-Lyzer from Sigma-Aldrich, both of which come in a variety of MWCO and volume ranges. While both dialysis cassettes work, for ease of use and availability, the Slide-A-Lyzer cassette was selected. There are many additional microdialysis, filtration, and buffer exchanging techniques and products available, which we did not explore. The products used in this work were selected because we

were not sample-limited and the aim was simply to verify the applicability of the dialysis HDX-MS approach.

Direct Comparison of the Dilution and Dialysis HDX Labeling Formats

In order to assess the viability of the dialysis HDX labeling approach, and to learn how this approach compares with the classical dilution labeling procedure, a head-to-head comparison was performed on mAb1 as follows. A 3 mg/mL stock solution of mAb1 was prepared and labeled at 4 °C by both dilution and dialysis HDX methods. This stock solution was then diluted approximately 15-fold in deuterated PBS buffer and incubated for 180, 360, and 840 min before quenching. For direct comparison against the dialysis labeling method, mAb1 was also diluted 15-fold in PBS buffer (no deuterium) to mimic the conditions experienced by the dilution method, and the sample was then placed into the minidialysis cartridge and labeled at 4 °C for 180, 360, and 840 min before quenching. This dilution step of mAb1 in dialysis workflow was performed to maintain a consistent concentration of labeled protein between both dilution and dialysis labeling formats. In total, more than 100 heavy chain (HC) and light chain (LC) peptides were identified and their deuterium levels were followed. The corresponding peptide coverage map is shown in the Supporting Information, Figure S1. Results of head-to-head comparison between dilution and dialysis formats are shown in Figure 2, where four representative mAb1 peptides were selected, two from the HC: residues 4-10 and 244-254; and two from the LC: residues 4-11, and residues 49-72. The data indicate that labeling by dialysis is equivalent to labeling by dilution, meaning that the rate of deuterium uptake follows a similar pattern in both labeling formats, as deduced from the red and blue traces in Figure 2. Most of the peptides in both HC and LC exhibited similar rates and extent of HDX, although several peptides showed slightly higher deuterium incorporation in the dialysis labeling (see Figure 2b). This is likely due to the fact that the dilution approach utilized approximately 15-fold labeling excess in D_2O, whereas the dialysis labeling approach contained several thousand-fold excess of D_2O in the beaker. Interestingly, even though the dialysis labeling format required extended labeling times (i.e., >3 h), the low temperature conditions slowed the chemical rate of HDX and the dynamics of the protein enough that some of the temporal aspects of labeling at 4 °C (i.e.,

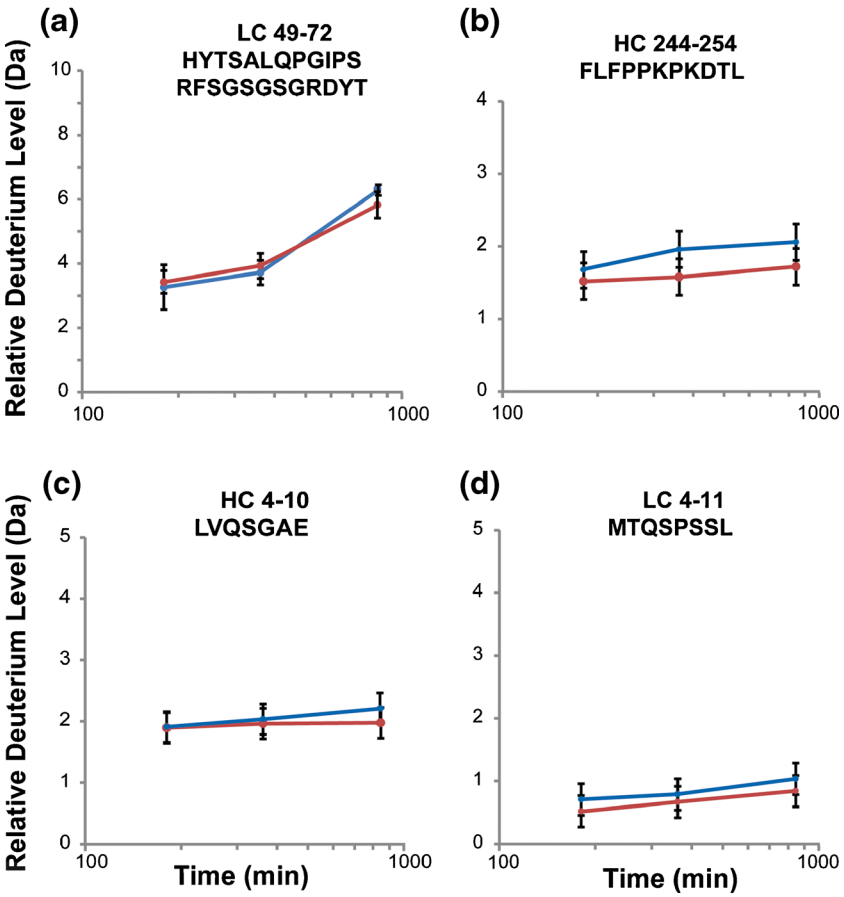

Figure 2. Relative deuterium uptake plots for four representative mAb1 peptides using two HDX approaches [dilution (red) and dialysis (blue)] at 4 °C. **(a)** Residues 244-254 from the Heavy Chain (HC), **(b)** residues 4-10 from the HC, **(c)** residues 4-11 from the Light Chain (LC), and **(d)** residues 49-72 from the Light Chain (LC). The obtained sequence coverage was 98.1% for LC and 88.2% for HC (see Supporting Information, Figure S1). All experiments were performed in triplicate. The deuterium uptake profile shows similar trend in both methods

dynamics) could still be captured, with several peptides showing incomplete HDX at the first time point (3 h), see Figure 2a.

It should be noted that low temperature HDX is not a requirement for this method; the analysis can be performed at any temperature. Furthermore, since the dialysis and dilution methods are performed differently (experimentally), the data from the dialysis HDX labeling procedure should only qualitatively resemble that from the dilution procedure. The main objective of these experiments is to compare samples labeled under similar conditions, thus all comparisons are relative. The data (whether it is from dialysis or dilution labeling procedures) simply needs to be reliable and robust within the given format, and this is evident from the replicate measurements performed (n = 3).

Preliminary Investigation of mAb Conformation, Dynamics, and Interactions at High Versus Low Concentration (Constant Mass Load)

The dialysis HDX labeling method was used to analyze the effect of high concentration on the conformation and dynamics of mAb1. mAb1 is an IgG4 with a molecular weight of approximately 148 kDa and a pI of 7.7. At high concentrations, mAb1 exhibits relatively high viscosity, approximately 50 cP at 200 mg/mL (see Supporting Information Figure S2). We expected that deuterium incorporation into the mAb upon labeling would not affect its viscosity. However, the viscosities of H_2O and D_2O are different (1.00 and 1.25 cP, respectively). Therefore, to confirm this we labeled mAb1 for 2 d by dialysis-HDX at ambient temperature and measured and compared the viscosity of the deuterated mAb1 solution at increasing concentrations to that of the undeuterated mAb1. As expected, the viscosity of the fully deuterated sample was comparable to the undeuterated sample at 25 °C (Supporting Information Figure S2). The increase in viscosity of mAb1 from 3 to 200 mg/mL was also not expected to influence the chemical HDX rate (k_{ch}) because it has been demonstrated in previous publications that decreased water activity and increased solution viscosity both had a negligible effect on HDX [25–27]. Thus, we assumed that a direct comparison could be made between the HDX-MS data acquired for mAb1 at 3 and 200 mg/mL concentration.

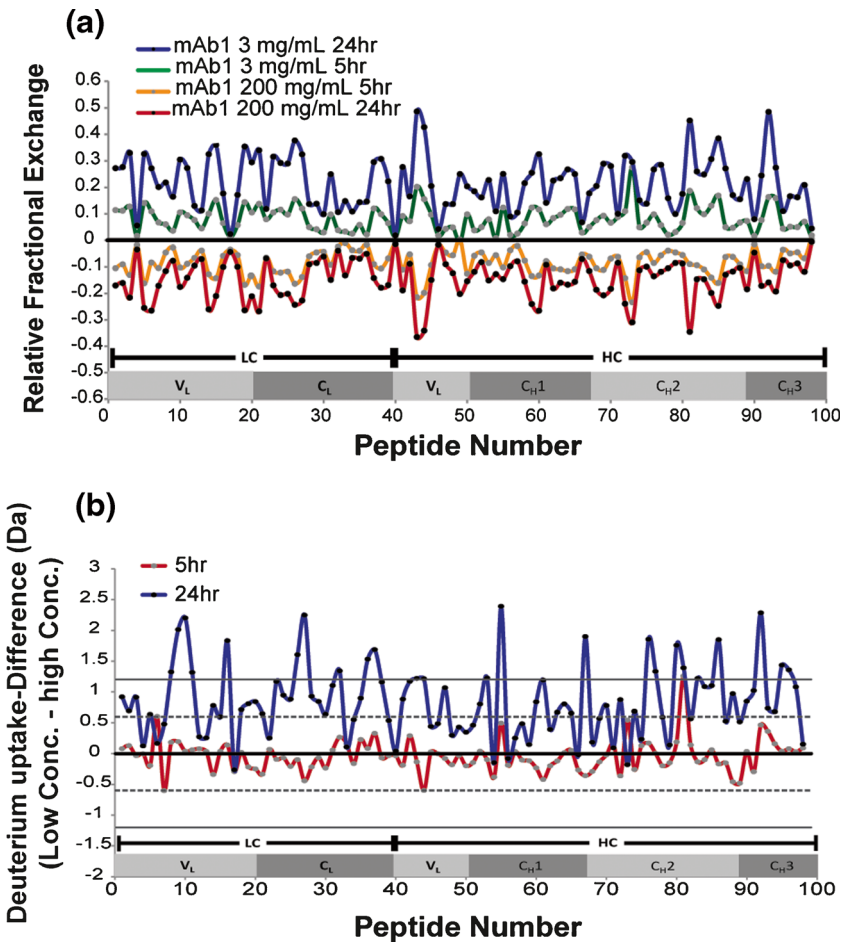

Figure 3. The butterfly plot of a set of >100 peptides identified in mAb1. **(a)** The deuterium contents were measured in both the 3 and 200 mg/mL samples and with labeling times of 5 h and 24 h, with the 3 mg/mL data on the top and the 200 mg/mL data on the bottom. The x-axis represents the peptides and the y-axis is the relative fractional exchange (e.g., a value of 0.5 relative fractional exchange corresponds to 50% exchanged). **(b)** The deuterium uptake data from the 3 mg/mL sample was subtracted from the data for 200 mg/mL sample in order to create a difference plot

As the next step, dialysis HDX-MS experiments were performed on mAb1 at 3 and 200 mg/mL to elucidate what differences could be detected in deuterium uptake profile of lower and higher concentrations. The HDX labeling was conducted at 4 °C (to lower the k_{ch} for the HDX reaction) and performed for 5 and 24 h, using the dialysis labeling approach as described earlier. After the allotted time, the samples were quenched and diluted such that the total on-column load was similar for all samples with a 100 µL injection volume. For the 3 mg/mL sample, 2 µL of sample was labeled or diluted with 25 µL of buffer. The sample was then quenched with 27 µL of quench buffer and quickly diluted 1:1 with 54 µL of 0.1% formic acid before injection. The time used to quench and dilute was carefully maintained to 1 min before injection, to ensure consistency in all samples. The total amount of injected protein was 5.6 µg; however, since there is always a small volume of sample (roughly 5 µL) that does not reach the column (due to sample fittings and line connections prior to the sample loop), we assume that the total amount of protein injected is approximately 5 µg. For the 200 mg/mL sample, the dilutions were scaled accordingly to ensure that the approximate amount of 5 µg of protein was injected and reached the column in a volume of 100 µL. Labeling of the 200 mg/mL sample required significant dilution to maintain 5 µg in 100 µL of injected sample; nevertheless, increased back-exchange was not observed (details associated with these experiments will be described in in forthcoming work).

In total, >100 peptides were reproducibly identified and their deuterium content was measured for both the 3 and 200 mg/mL samples (see Supporting Information Figure S1 for the coverage map). All of the HDX data was plotted onto a single butterfly graph (the details associated with this representation are described elsewhere [28]), with the 3 mg/mL data on the top and the 200 mg/mL data on the bottom (Figure 3a). In Figure 3a, the x-axis represents all of the peptides monitored and the y-axis is the relative fractional exchange (e.g., a value of 0.5 relative fractional exchange corresponds to 50% exchanged). Such a representation allows one to view the temporal or dynamic aspect of the protein in different states at the same time and to identify the regions in the protein structure that exchange very little and those that exchange more readily.

The deuterium uptake data from the low and highly concentrated samples was then subtracted in order to create an HDX difference plot (Figure 3b). In this plot, the difference is represented as an absolute mass difference, which is expressed in Da. If no difference exists between the samples, all points should have a y-axis value of zero (or close to zero, within the preset threshold, which for these data is ±0.6 Da, based on replicate injections). At the 5-h time point, most of the HDX data is comparable between 3 and 200 mg/mL samples, suggesting that the overall conformational dynamics of the mAb is similar in both concentrations during the 5-h labeling period. Only one peptide in both HC and LC was observed to have a significant difference at 5 h of exchange, HC peptide 322-336 (shown in Figure 4a). However, more significant differences were observed at 24 h of exchange, with more than 40% of the entire mAb1 protein affected, see Table 1. The difference plot (Figure 3b) shows that most of the peptides in 3 mg/mL solution have a positive difference value, which implies that the 3 mg/mL sample contains a greater level of deuterium relative to the 200 mg/mL sample.

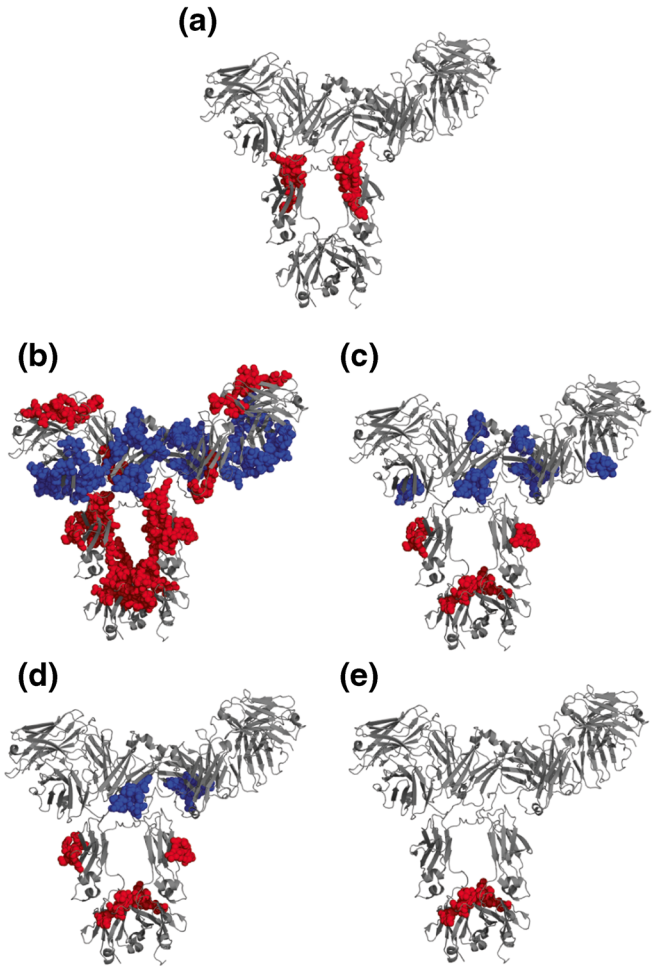

Figure 4. **(a)** The difference in HDX mapped to a homology model (based on PDB: 1HZH) of mAb1 at 24 h HDX labeling time point. Differences associated with the HC and LC are shown in red and blue spheres, respectively. **(b)** Regions with ≥10% difference, **(c)** regions with between 11% and 15% difference, **(d)** regions with >15% difference, and **(e)** regions with >20% relative deuterium change are demonstrated. Differences were detected in both the Fab and Fc and in both HC and LC however, the region most significantly affected is the Fc region

Table 1. Relative Differences in HDX Observed Between mAb1 at 3 and 200 mg/mL with 24-h labeling at 4 °C

mAb1	All peptides with differences > 1 Da Relative effect	All peptides with differences > 10% Relative effect
LC	59%	45%
HC	31%	21%
Total	40%	29%

These data can also be depicted as a relative percent change on a representative protein structure (Figure 4). The difference in HDX was mapped to a homology model of mAb1 built from PDB: 1HZH. In Figure 4, mAb1 is shown as a grey cartoon structure where the differences in deuterium uptake in the HC and LC are shown in red and blue spheres, respectively. Those regions with a change in the relative deuterium incorporation between the 3 and 200 mg/mL sample ≥10% are shown in Figure 4b, those with a change between 11% and 15% are shown in Figure 4c, those with >15% change are shown in Figure 4d, and those with a >20% change are shown in Figure 4e. Differences were detected in both the Fab and Fc and in both HC and LC; however, we found that the Fc region was most significantly affected. These observations indicate that the level of deuterium incorporation decreased with an increase in concentration, suggesting a change in the mAbs dynamic behavior.

Several explanations present themselves for the observed local reductions in dynamics of the mAb in the higher concentration sample. One possibility is that the regions of mAb1 protected from HDX at high protein concentration are directly involved in specific intermolecular interactions with neighboring mAb1 molecules and such interactions shield these regions from exchange or stabilize existing hydrogen bonding networks. A second possibility is that the elevated concentration results in crowding effects, which could influence how neighboring proteins interact and/or alter the free motion of individual mAb1 domains, essentially "tightening" or "loosening" the mAbs inter-domain motions. A third possibility is that a combination of these phenomena plays a role in altering dynamics of the protein at high concentrations. It is possible that the time scale for this experiment may be too long to observe and/or distinguish between any of the phenomena mentioned above. Nevertheless, changes in HDX in the EX2 time regime, which constitutes a shift in the average hydrogen bonding status of the equilibrium ensemble of protein molecules in solution, have been reproducibly observed between proteins/ligands that bind very weakly (i.e., K_d in the high μM to low mM range) [29]. It is also worth noting that no crowding and/or viscosity-induced unfolding was observed, which would be an important marker for aggregation propensity in biopharmaceutical formulations (though admittedly not a precondition for aggregation).

Unfortunately, HDX-MS (in general) is not able to unequivocally map interaction sites, but rather, in this work, maps sites with perturbed dynamics, between mAb molecules at high concentration. Regardless of the specific mechanism(s) involved, the changes we observe at relatively long labeling times indicate (reproducibly) that even very stable hydrogen bonds of the mAb are perturbed by "concentration effects." Hence, we are convinced that such information provides a unique direct measure of the conformational impact of the sum of high concentration effects occurring in solution. In combination with other orthogonal biophysical measurements, HDX-MS measurements can likely be very useful in understanding the causes of high viscosity in highly concentrated samples. Future work will continue to develop the dialysis HDX-MS approach, and more insights can likely be gained by comparison of a wider selection of different yet related mAbs.

Conclusion

Classical continuous HDX labeling via dilution is not applicable in analysis of highly concentrated protein solutions (i.e., ≥ approximately 40 mg/mL). Although there are alternative labeling formats (i.e., by dilution of lyophilized product [11]), we were interested in labeling a highly concentrated protein in an equilibrated solution state with minimal sample manipulation. This was achieved with the development of a dialysis HDX labeling format using minidialysis cartridges, at lower labeling temperatures (although lower temperatures may not be necessary), and longer labeling times (hours, to accommodate the time needed for dialysis to reach equilibrium). The dilution and dialysis HDX labeling formats were compared and were shown to be similar at low temperature labeling. We also demonstrated that while the labeling times were significantly longer, HDX kinetics and dynamics of the protein could still be monitored reproducibly. Moreover, although further experiments are needed to optimize the method, HDX labeling by dialysis appears to show promise in studying proteins at high concentrations. In addition to investigating the viability of the dialysis-HDX method, we also analyzed the effect of increased concentration on deuterium uptake profile of an IgG4 mAb (mAb1) at two different concentrations (3 and 200 mg/mL). The results from this and our ongoing work may provide insight and improve our understanding of the behavior of mAbs at high concentration formulations.

Acknowledgments

The authors thank Jessica Ballinger for her continued support and Dr. Steven Berkowitz for his helpful discussions and advice. We would also like to thank the PhD school of the Faculty of Health and Medical Sciences at the University of Copenhagen for financial support and a PhD fellowship, as well, we gratefully acknowledge the financial support from The Marie Curie Actions Programme of the E.U (Grant No. PCIG09-GA-2011-294214) and the Danish Council for Independent Research|Natural Sciences (Steno Grant No. 11-104058).

References

1. Adler, M.J., Dimitrov, D.S.: Therapeutic antibodies against cancer. Hematol. Oncol. Clin. North Am **26**, 447–481 (2012)
2. Chames, P., Van Regenmortel, M., Weiss, E., Baty, D.: Therapeutic antibodies: successes, limitations, and hopes for the future. Br. J. Pharmacol. **157**, 220–233 (2009)
3. Daugherty, A.L., Mrsny, R.J.: Formulation and delivery issues for monoclonal antibody therapeutics. Adv. Drug Deliv. Rev. **58**, 686–706 (2006)
4. Shire, S.J., Shahrokh, Z., Liu, J.: Challenges in the development of high protein concentration formulations. J. Pharm. Sci. **93**, 1390–1402 (2004)
5. Neergaard, M.S., Kalonia, D.S., Parshad, H., Nielsen, A.D., Møller, E.H., van de Weert, M.: Viscosity of high concentration protein formulations of

monoclonal antibodies of the IgG1 and IgG4 subclass—prediction of viscosity through protein–protein interaction measurements. Eur. J. Pharm. Sci. **49**, 400–410 (2013)
6. Yadav, S., Shire, S.J., Kalonia, D.S.: Factors affecting the viscosity in high concentration solutions of different monoclonal antibodies. J. Pharm. Sci. **99**, 4812–4829 (2010)
7. Harris, R.J., Shire, S.J., Winter, C.: Commercial manufacturing scale formulation and analytical characterization of therapeutic recombinant antibodies. Drug Dev. Res. **61**, 137–154 (2004)
8. Cromwell, M.M., Hilario, E., Jacobson, F.: Protein aggregation and bioprocessing. AAPS J. **8**, E572–E579 (2006)
9. Wales, T.E., Engen, J.R.: Hydrogen exchange mass spectrometry for the analysis of protein dynamics. Mass Spectrom. Rev. **25**, 158–170 (2006)
10. Engen, J.R., Wales, T.E.: Analytical aspects of hydrogen exchange mass spectrometry. Annu. Rev. Anal. Chem. **8**, 127–148 (2015)
11. Arora, J., Hickey, J.M., Majumdar, R., Esfandiary, R., Bishop, S.M., Samra, H.S., Middaugh, C.R., Weis, D.D., Volkin, D.B.: Hydrogen exchange mass spectrometry reveals protein interfaces and distant dynamic coupling effects during the reversible self-association of an IgG1 monoclonal antibody. mAbs. **7**, 525–539 (2015)
12. Kasper, J.C., Winter, G., Friess, W.: Recent advances and further challenges in lyophilization. Eur. J. Pharm. Biopharm. **85**, 162–169 (2013)
13. Carpenter, J.F., Prestrelski, S.J., Arakawa, T.: Separation of freezing- and drying-induced denaturation of lyophilized proteins using stress-specific stabilization: I. Enzyme activity and calorimetric studies. Arch. Biochem. Biophys. **303**, 456–464 (1993)
14. Carpenter, J.F., Chang, B.S., Garzon-Rodriguez, W., Randolph, T.W.: Rational design of stable lyophilized protein formulations: theory and practice. Springer, New York, NY (2002)
15. Chang, B.S., Kendrick, B.S., Carpenter, J.F.: Surface-induced denaturation of proteins during freezing and its inhibition by surfactants. J. Pharm. Sci. **85**, 1325–1330 (1996)
16. Morris, G.J., Goodrich, M., Acton, E., Fonseca, F.: The high viscosity encountered during freezing in glycerol solutions: effects on cryopreservation. Cryobiology **52**, 323–334 (2006)
17. Ybema, H., Kolkman-Roodbeen, L., te Booy, M.P., Vromans, H.: Vial lyophilization: calculations on rate limitation during primary drying. Pharm. Res. **12**, 1260–1263 (1995)
18. Astorga-Wells, J., Landreh, M., Johansson, J., Bergman, T., Jörnvall, H.: A membrane cell for on-line hydrogen/deuterium exchange to study protein folding and protein–protein interactions by mass spectrometry. Mo. Cell. Proteom **10**(M110), 006510 (2011)
19. Houde, D., Engen, J.R.: Conformational Analysis of Recombinant Monoclonal Antibodies with Hydrogen/Deuterium Exchange Mass Spectrometry. In: Beck, A. (Ed.). Humana Press, New York, NY (2013)
20. Fang, J., Rand, K.D., Beuning, P.J., Engen, J.R.: False EX1 signatures caused by sample carryover during HX MS analyses. Int. J. Mass Spectrom. **302**, 19–25 (2011)
21. Plumb, R.S., Johnson, K.A., Rainville, P., Smith, B.W., Wilson, I.D., Castro-Perez, J.M., Nicholson, J.K.: UPLC/MSE: a new approach for generating molecular fragment information for biomarker structure elucidation. Rapid Commun. Mass Spectrom. **20**, 1989–1994 (2006)
22. Seeliger, D., de Groot, B.: Ligand docking and binding site analysis with PyMOL and Autodock/Vina. J. Comput. Aided Mol. Des. **24**, 417–422 (2010)
23. Thirumangalathu, R., Krishnan, S., Ricci, M.S., Brems, D.N., Randolph, T.W., Carpenter, J.F.: Silicone oil- and agitation-induced aggregation of a monoclonal antibody in aqueous solution. J. Pharm. Sci. **98**, 3167–3181 (2009)
24. Fesinmeyer, R.M., Hogan, S., Saluja, A., Brych, S.R., Kras, E., Narhi, L.O., Brems, D.N., Gokarn, Y.R.: Effect of ions on agitation-and temperature-induced aggregation reactions of antibodies. Pharm. Res. **26**, 903–913 (2009)
25. Manikwar, P., Majumdar, R., Hickey, J.M., Thakkar, S.V., Samra, H.S., Sathish, H.A., Bishop, S.M., Middaugh, C.R., Weis, D.D., Volkin, D.B.: Correlating excipient effects on conformational and storage stability of an IgG1 monoclonal antibody with local dynamics as measured by hydrogen/deuterium-exchange mass spectrometry. J. Pharm. Sci. **102**, 2136–2151 (2013)
26. Wang, A., Robertson, A.D., Bolen, D.: Effects of a naturally occurring compatible osmolyte on the internal dynamics of ribonuclease A. Biochemistry **34**, 15096–15104 (1995)
27. Lim, W.K., Rösgen, J., Englander, S.W.: Urea, but not guanidinium, destabilizes proteins by forming hydrogen bonds to the peptide group. Proc. Natl. Acad. Sci. **106**, 2595–2600 (2009)
28. Houde, D., Berkowitz, S.A., Engen, J.R.: The utility of hydrogen/deuterium exchange mass spectrometry in biopharmaceutical comparability studies. J. Pharm. Sci. **100**, 2071–2086 (2011)
29. Leurs, U., Lohse, B., Ming, S., Cole, P.A., Clausen, R.P., Kristensen, J.L., Rand, K.D.: Dissecting the binding mode of low affinity phage display peptide ligands to protein targets by hydrogen/deuterium exchange coupled to mass spectrometry. Anal. Chem. **86**, 11734–11741 (2014)